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Disclosure of interest

The authors declare that they have no competing interest.

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Abstract

Background: Emergence of coronavirus disease 2019 (COVID-19) is a major healthcare threat. Apparently, the novel coronavirus (SARS-CoV-2) is armed by special abilities to spread and dysregulate the immune mechanisms. The likelihood of oropharyngeal candidiasis (OPC) development in COVID-19 patients with a list of attributable risk factors for oral infections has not yet been investigated.

Objectives: We here aim to investigate the prevalence, causative agents, and antifungal susceptibility pattern of OPC in Iranian COVID-19 patients.

Patients and Methods: A total of 53 hospitalized COVID-19 patients with OPC were studied. Relevant clinical data were mined. Strain identification was performed by 21-plex PCR and sequencing of the internal transcribed spacer region (ITS1-5.8S-ITS2). Antifungal susceptibility testing to fluconazole, itraconazole, voriconazole, amphotericin B, caspofungin, micafungin and anidulafungin was performed according to the CLSI broth dilution method.

Results: In 53 COVID-19 patients with OPC, cardiovascular diseases (52.83 %), and diabetes (37.7 %) were the principal underlying conditions. The most common risk factor was lymphopenia (71%). In total, 65 *Candida* isolates causing OPC were recovered. *C. albicans* (70.7%) was the most common, followed by *C. glabrata* (10.7%), *C. dubliniensis* (9.2%), *C. parapsilosis* sensu stricto (4.6%), *C. tropicalis* (3%), and *Pichia kudriavzevii* (=*C. krusei*, 1.5%). Majority of the *Candida* isolates were susceptible to all three classes of antifungal drugs.

Conclusion: Our data clarified some concerns regarding the occurrence of OPC in Iranian COVID-19 patients. Further studies should be conducted to design an appropriate prophylaxis program and improve management of OPC in critically ill COVID-19 patients.

Keywords: Oral candidiasis, COVID-19, Coinfection, Oropharyngeal candidiasis

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Introduction

Since December 2019, an unprecedented outbreak of viral pneumonia caused by an initially unknown viral pathogen linked to a seafood associated wholesale market emerged in Wuhan, Hubei Province, China [1, 2]. The pathogen of the disease was soon identified as a novel coronavirus (SARS-CoV-2), and the disease was named coronavirus disease-19 (COVID-19) [3]. Despite global containment and quarantine attempts, the incidence continued to increase, spread to many other countries and caused a pandemic with a great number of deaths [4]. The mortality rate differs greatly from country to country [5]. Among various factors leading to morbidity and mortality in COVID-19 patients, the prevalence and role of bacterial and fungal co-infections has not yet been discussed, particularly in patients suffering from acute respiratory distress syndrome (ARDS). So far, inadequate attention has been given to the prevalence of fungal infections in patients suffering from COVID-19 that may experience lymphocytopenia, hospitalization in intensive care unit (ICU), broad-spectrum antibiotics and corticosteroid usage, intubation, cytokine storms, and having underlying diseases which make them severely immunocompromised [5-9]. Due to undefined pharmacological treatment for COVID-19, indirect complex effect, invasive therapeutic methods and multi-drug treatment, some pathological oral conditions can be expected to be aggravated by SARS-CoV-2, particularly in those patients with a compromised immune mechanism, or that take long-term pharmacotherapies [10]. For these reasons they are at substantial risk for developing mucosal candidiasis. Based on our centers experiences in the management of severely COVID-19 patients, oropharyngeal candidiasis (OPC) might be a probable cause of morbidity in these patients that begins with colonization of the *Candida* species on the oral mucosa. Consequently, local discomfort, an altered taste sensation, oral burning, glossodynia, dysphagia and difficulty in breathing may be felt by the patients [11]. In the majority of cases this opportunistic yeast infection is endogenously acquired and develops when local host defenses are weakened [12, 13]. *Candida albicans* is the most important species (>80%) that causes OPC [9].

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Nonetheless, non-*albicans* species, such as *C. tropicalis*, *C. glabrata, C. parapsilosis*, *Pichia kudriavzevii* (=*C. krusei*) and *C. dubliniensis,* are also known to contribute to this infection [9]. In case of untreated, ineffective treatment, OPC caused by fluconazole-resistant *Candida* species or in patients with an immunocompromised status, the infection can regionally spread from the oropharynx to the esophagus or systematically through the bloodstream or upper gastrointestinal tract leading to candidemia with significant morbidity and mortality [11]. Hence, timely detection of OPC and accurate identification of etiological agents in patients suffering from COVID-19 are important to optimize effective therapy and improvement of outcome. So far, the likelihood of OPC development, as the most prevalent mucocutaneous mycosis of the oral cavity in severe COVID-19 patients has not yet been investigated. Therefore, the current study was conducted to investigate the prevalence, causative agents, and antifungal susceptibility pattern of OPC in Iranian COVID-19 patients.

Materials and Methods

Study design, patients and specimens

This cross-sectional study was undertaken from March 1, 2020 to April 30, 2020 on all patients with clinically and laboratory confirmed COVID-19 infections at three tertiary care training hospitals (Imam Khomeini hospital complex, Ziaeian hospital, and Hazrat Rasoul Akram hospital) affiliated with Tehran University of Medical Sciences and Iran University of Medical Sciences, Tehran, Iran. Verbal consent was obtained from patients before being enrolled in this study. The protocol of this study was in accordance with the principals established by the Declaration of Helsinki and was approved by the ethics committee of Tehran University of Medical Sciences, Tehran, Iran (IR.TUMS.VCR.REC.1399.058). The oral cavity of patients was examined for OPC and those with clinically-confirmed OPC representing by finding pseudomembranous structures or white plaques on the intraoral mucous layer, with or without other complaints such as dry mouth and glossalgia were included in this study. The demographic and clinical data were documented in the patients' sheets. Sampling was carried out from oral plaques using sterile swabs. OPC was confirmed by the presence of budding yeasts and pseudohyphae in KOH 10% preparation and culture. The swabs were streaked on Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich) plates on bedside. Plates were incubated at 37 °C and checked daily.

Phenotypic and molecular identification

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For obtaining pure single colonies and preliminary identification, the grown yeast isolates were subcultured on CHROMagar Candida medium (CHROMagar, Paris, France). Accurate identification of isolates was based on a 3-step 21-plex PCR method using primers and conditions described previously [14, 15]. A set of standard strains of common *Candida* species were included as controls. To identify isolates with inconclusive results in the 21-plex PCR, the ITS1-5.8S rDNA-ITS2 region was amplified using primers ITS1: 5'-TCCGTAGGTGAACCTGCGG-3 and ITS4: 5'–TCCTCCGCTTATTGATATGC-3 using conditions described previously [16]. PCR amplicons were sequenced and results were analyzed using NCBI BLAST (https://www.blast.ncbi.nlm.nih.gov/Blast.cgi) and ISHAM barcoding (http://its.mycologylab.org) databases. The ITS1-5.8S rDNA-ITS2 sequences of isolates have been deposited in the GenBank (For example an accession number for each species; MT640021 to MT640028).

Antifungal susceptibility testing

Antifungal susceptibility patterns of isolates to three classes of antifungal drugs, i.e. azoles (fluconazole, voriconazole, and itraconazole), polyenes (amphotericin B), and echinocandins (caspofungin, anidulafungin, and micafungin) were assessed according to the fourth edition of the Clinical and Laboratory Standards Institute M27 standard method [17]. Stock solutions of drugs were prepared in DMSO and diluted in RPMI-1640 medium (Gibco, NY, USA) to obtain 2x the final concentrations. The final concentrations ranged from 0.015 to 8 µg/mL for echinocandins, 0.031 to 16 µg/mL for amphotericin B, itraconazole and voriconazole, and 0.125 to 64 µg/mL for fluconazole. Hundred µLs of serial dilutions of drugs were dispensed into columns 1 to 10 of 96-well microtiter plates and the plates were stored at -80 °C until their use. Fungal inoculum was prepared using fresh colonies and adjusted to a transmittance within the range 75-77% at 530 nm wavelength. Resulting suspensions were diluted 1:20 and then 1:100 in RPMI-1640 medium to obtain working inoculums. Subsequently, 100 µL of suspensions were added to all wells of test plates except for the column 11 which was assigned as inoculum-free negative control. Column 12 was used as drug-free growth control. Plates were incubated at 35 °C and results were visually read after 24 hours in comparison to growth control wells. *C. parapsilosis* (ATCC 22019) and *Pichia kudriavzevii* (ATCC 6258) standard strains were used as quality control. The minimum inhibitory concentrations (MICs) were interpreted according to the breakpoints or epidemiological cut-off values (ECV) provided in CLSI M60 and

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M59 supplements [18, 19]. Results of itraconazole against *C. albicans, C. dubliniensis*, and *C. parapsilosis* were interpreted according to the values established by Pfaller *et al*. [20].

Statistical analysis

All statistical analyses were performed using SPSS v24 (SPSS Inc., Chicago, IL, USA). Descriptive test was performed to describe the demographic characteristics and Chi-Square test was performed on all variables of this study. Statistical significance was assumed with $p = 0.05$.

Results

Patient characteristics

During the period of this study, 53 (5%) out of 1059 Iranian patients with confirmed COVID-19 infection had OPC. The patients were within an age range of 27 to 90 years with a mean ±SD of 63.1 ± 16.4 years. Almost 80% of the patients (n=42) were ≥50 years of age, which was significantly associated with OPC (p = 0.03). Females (30/53; 56.6%) were slightly more affected than males (43.4%) by OPC. The mean ±SD time interval between diagnosis of COVID-19 and clinical presentations of OPC leading to specimen collection was 8 days (range: 1–30 days) (Figure 1). Infectious ward (15/53; 28.3%) and Thorax ward (12/53; 22.6%) contained the highest number of patients. The demographics and background clinical data of patients are shown in Table 1. Cardiovascular diseases (28/53; 52.8%), and diabetes (20/53; 37.7 %) were the principal underlying conditions in COVID-19 patients with OPC. Seventy-one percent of patients suffering COVID-19 infection with OPC showed lymphopenia (a median lymphocyte count of 1000 cells/mm) (P < 0.001). Due to the simultaneous occurrence of COVID-19 infection and OPC, various drugs including antiviral (53/53; 100%), antibacterial (49/53; 92.5%) and antifungal (52/53; 98.1%) as well as corticosteroids (25/53; 47.1%) were administered. Methylprednisolone (18/53; 33.9 %) was the main corticosteroid prescribed to patients, followed by hydrocortisone (5/53; 9.4 %), and dexamethasone (4/53; 7.5 %). Regarding antifungal drugs, except for 1 (1.8%) patient who did not receive any treatment, 21 (39.6%), 13 (24.5%), and 1 (1.8%) patients were treated by fluconazole, nystatin, and caspofungin, respectively. The remaining 17 (32%) patients were given a combination of fluconazole and nystatin. The mean ±SD duration of antifungal treatment was 4.79 ±2.11 days.

Distribution of Candida species

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In total 65 *Candida* isolates causing OPC were recovered from 53 patients. Mixed infections were observed in 9 cases (6 cases of coinfection by two and 3 cases by three *Candida* species). *C. albicans* (46/6; 70.7%) was the most prevalent yeast species. Among the main non-*albicans Candida* (NAC) species, *C. glabrata* (7/65; 10.7%) was the predominant species, followed by *C. dubliniensis* (6/65; 9.2%), *C. parapsilosis* sensu stricto (3/65; 4.6%), *C. tropicalis* (2/65; 3%), and *P. kudriavzevii* (1/65; 1.5%).

Antifungal susceptibility testing

The distribution of *Candida* isolates based on the MIC values of antifungal drugs, the MIC ranges, MIC_{50}, geometric mean (GM) and percentage of susceptible/wild-type isolates are shown in Table 2. In general, there was a high level of susceptibility to all the tested antifungal drugs. Amphotericin B (overall MIC range: 0.016–0.25 µg/mL, GM: 0.027 µg/mL, MIC_{50}: 0.016 µg/mL), anidulafungin (overall MIC range: 0.008–0.062 µg/mL, GM: 0.014 µg/mL, MIC_{50}: 0.016 µg/mL), and micafungin (overall MIC range: 0.008–0.016 µg/mL, GM: 0.009 µg/mL, MIC_{50}: 0.008 µg/mL) were the most active drugs and no case of resistance to these antifungal drugs was noted. The overall narrowest and widest MIC ranges were observed for anidulafungin and micafungin (0.008–0.016 µg/mL) and caspofungin (0.008–2 µg/mL), respectively. All isolates were susceptible/wild-type to fluconazole (overall MIC range: 0.125–1 µg/mL), except for one *P. kudriavzevii* isolate (MIC: 4 µg/mL), a species that is intrinsically resistant, and one isolate of *C. dubliniensis* (MIC: 2 µg/mL). For voriconazole, two out of 46 (4.35%) *C. albicans* isolates were intermediate, while the remaining isolates were susceptible. Caspofungin was the least active drug. The only isolate of *P. kudriavzevii* (MIC: 2 µg/mL) and one isolate of *C. dubliniensis* (MIC: 1 µg/mL) were caspofungin-resistant. Furthermore, two out of 46 (4.35%) *C. albicans* isolates, and all *C. glabrata* isolates were intermediate to this drug.

Discussion

An enveloped novel coronavirus caused a global pandemic burden with life-threatening outcomes [7, 10, 21]. Apparently, the virus is armed by special abilities to spread and dysregulate the immune mechanisms [22]. Several studies have addressed the probability of occurrence of fungal co-infections, particularly invasive pulmonary aspergillosis in COVID-19 patients [5, 23-29]. Furthermore, due to several risk factors, COVID-19 patients are also vulnerable to infections caused by the emerging

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species, *C. auris* [30]. During our multicenter experiences in the management of patients with COVID-19, we found that pseudomembranous white plaques and erythematous areas occurred in the oral mucosa of 5 % of hospitalized patients with COVID-19, and on average occurred within 8 days of onset of COVID-19. Our data showed that OPC is more probably to infect older adult COVID-19 patients with cardiovascular diseases and diabetes status as a result of weaker immune functions of these patients. Similarly, in previous studies, cerebrovascular diseases and diabetes were the main reported chronic underlying diseases in patients with COVID-19, which were more common among patients with severe disease and were associated with poor prognosis [6, 31]. We found that increasing age (≥50 years) was significantly associated with OPC among COVID-19 patients. In a cross-sectional study, Suryana *et al*. revealed that increasing age (P = 0.03) was significantly identified risk factor in HIV patients [32]. Elderly patients were shown to have significantly lower activity levels of protective salivary innate defenses [33]. Considering the clinical course, disease progression and severity of COVID-19, most of COVID-19 patients with critically ill conditions inevitably experience at least one of following risk factors, for OPC, including lymphocytopenia, ICU admission, invasive or non-invasive ventilation, corticosteroid and broad spectrum antibiotics usage or having immunocompromised condition which give them a significantly increased risk for the development of opportunistic fungal infections [5, 6, 11, 22, 34]. In our descriptive study, broad-spectrum antibiotic therapy, lymphocytopenia, ICU admission, systemic corticosteroids usage and mechanical ventilation were documented as the most frequent factors predisposed our subjects with COVID-19 to develop OPC. However, these findings need to be verified by a comparative case control study in the future to accurately identify the risk factors for OPC in patients with COVID-19. Broad spectrum antibiotics usage was the most associated risk factors for the development of OPC in COVID-19 patients as it occurred in nearly 92.5 of our subjects. Dysbiosis by bacterial depletion due to the use of broad-spectrum antibiotics can alter the local oral flora, creating a favorable environment for *Candida* to proliferate [35]. In a study from Chinese hospitals, the usage rate of antibiotics and antifungal agents in patients with severe COVID-19 were 100% and 39 %, respectively [36]. It is noteworthy that lymphocyte counts were found to be below the normal range in nearly 63-85% of patients with COVID-19 indicating lymphocytopenia (approximately 63-83%) as the main laboratory finding [6, 7, 22, 31]. The possible reason for the high rate of OPC in lymphocytopenic patients can be attributed to

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lymphocytes consumption by the virus; especially T lymphocytes as was also documented in infections caused HIV and SARS-CoV resulting in a substantial decrease of the total number of lymphocytes and, subsequently, result in an immunocompromised status of the patients [10, 22, 37]. A former study has shown that 16% of COVID-19 patients were admitted to the ICU and 8.3% underwent invasive ventilation [6]. Du and coworkers reported that 39% of superinfections were evidenced in COVID-19 patients hospitalized in ICUs in China [36]. Different groups of anti-inflammatory medications for treatment of patients with severe COVID-19 pneumonia are frequently used, although with conflicting information regarding their efficacy [7, 10]. Oral exposure to topical or systemic corticosteroids is another common cause of medication associated oral candidiasis, probably due to alterations of the local mucosal immunity [9, 11, 38]. In agreement with previous studies [6, 7], 47.2 % of our COVID-19 patients with OPC had a history of corticosteroids use. In the current study, *C. albicans* (70%) and NAC species (30%) were isolated from COVID-19 patients with OPC. These data are in agreement with the findings of previous OPC reports [35, 39, 40]. NAC isolates are remarkable pathogenic agents involved in OPC, which is of relevance to decide on the selection of therapeutics for this infection [35, 41]. Interestingly, a high percentage of C. *dubliniensis* was observed in our patients, which is similar to those reported in OPC caused by NAC species in HIV infected patients [37, 39]. In general, there were a high level of susceptibility to all the tested antifungal drugs. The increasing use of fluconazole to treat OPC and the emergence of azole resistance has resulted in a change in the prevalence of different *Candida* species [42]. Long term use of azoles may lead to the selection of less sensitive species, such as *P. kudriavzevii, C. dubliniensis,* and *C. glabrata,* and the development of resistance in previously susceptible *Candida* strains [37]. In conclusion, our data clarified some concerns regarding the occurrence of OPC in Iranian COVID-19 patients. Data from our centers can contribute to decide on more effective strategies in antifungal treatments and to design an appropriate prophylaxis program for the benefit of such patients.

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Figure legends

Figure 1. Hospitalized time between diagnosis of COVID-19 and clinical presentations of OPC

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Table 1. Demographic and clinical characteristics of patients with Iranian COVID-19 infection and oropharyngeal candidiasis

Variables	Frequency	Percentage
Gender		
Male	23	43.4
Female	30	56.6
Age groups		
<50	11	20.7
≥50	42	79.3
Underlying conditions		
Cardiovascular diseases	28	52.8
Diabetes	20	37.7
Chronic kidney diseases	11	20.7
Hematological malignancies	5	9.4
Risk factors		
Recipient broad-spectrum antibiotics	49	92
Corticosteroid therapy	25	47
Admission to ICU	26	49
Mechanical ventilation	16	30
Respiratory support		
Non-invasive	49	92.4
Invasive	4	7.5
Clinical presentations		
Lymphopenia	38	71.7
Leukopenia	10	18.9
Leukocytosis	10	18.9
Prolonged fever	39	73.5
Respiratory distress	50	94.3

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Table 2. *In vitro* antifungal susceptibility pattern of *Candida* isolates recovered from oropharyngeal lesions of Iranian COVID-19 patients

Candida species (n)	Antifungal drugs	Distribution of isolates based on MIC values (µg/mL)										MIC range (µg/mL)	MIC_{50} (µg/mL)	GM (µg/mL)	% of S/WT
		0.008	0.016	0.031	0.062	0.125	0.25	0.5	1	2	4				
C. albicans (46)	FLU					8	**25**[a]	10	2	1		0.125–2	0.25	0.286	100
	ITR		1	3	11	**22**	8	1				0.016–0.5	0.125	0.107	80.43
	VRC		6	**19**	16	3	2					0.016–0.25	0.031	0.043	95.65
	AmB		**27**	11	6	1		1				0.016–0.5	0.016	0.025	100
	CAS	2	5	**16**	14	6	1	2				0.008–0.5	0.031	0.048	95.65
	MCF	**41**	5									0.008–0.016	0.008	0.009	100
	ANI	**21**	15	7	3							0.008–0.062	0.016	0.014	100
C. glabrata (7)	FLU							**4**	1	2		0.5–2	0.5	0.82	100
	ITR				1	**2**	**2**	1	1			0.062–1	0.25	0.226	100
	VRC			**3**	**3**	1						0.031–0.125	0.062	0.051	100
	AmB		**3**	1	**3**							0.031–0.125	0.031	0.031	100
	CAS						**7**					0.25	0.25	0.25	0
	MCF	**7**										0.008	0.008	0.008	100
	ANI	**3**	2	2								0.008–0.031	0.016	0.014	100
C. dubliniensis [b] (6)	FLU					**3**	2			1		0.125–2	0.125	0.25	83.33
	ITR		1		1	**4**						0.016–0.125	0.125	0.079	100
	VRC		2		**4**							0.016–0.062	0.062	0.039	100
	AmB		2	**3**			1					0.016–0.25	0.031	0.035	100
	CAS		**3**	1	1				1			0.016–1	0.016	0.044	83.33
	MCF	**5**	1									0.008–0.016	0.008	0.009	100
	ANI	**4**	1	1								.0008–0.031	0.008	0.011	100
C. parapsilosis (3)	FLU							3				0.5	0.5	0.5	100
	ITR			3								0.031	0.031	0.031	100
	VRC			3								0.031	0.031	0.031	100
	AmB			3								0.031	0.031	0.031	100
	CAS						3					0.25	0.25	0.25	100
	MCF	3										0.008	0.008	0.008	100

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	ANI	3											0.008	0.008	0.008	100
C. tropicalis (2)	FLU							2					0.5	-	-	100
	ITR					2							0.125	-	-	100
	VRC			1		1							0.031–0.125	-	-	100
	AmB		1		1								0.016–0.062	-	-	100
	CAS					2							0.125	-	-	100
	MCF	1	1										0.008–0.016	-	-	100
	ANI		1	1									0.016–0.031	-	-	100
Pichia kudriavzevii (= *C. krusei*) (1)	FLU											1	-	-	-	0[c]
	ITR						1						-	-	-	100
	VRC					1							-	-	-	100
	AmB				1								-	-	-	100
	CAS									1			-	-	-	0
	MCF		1										-	-	-	100
	ANI				1								-	-	-	100

Abbreviations: MIC minimum inhibitory concentration, FLU fluconazole, ITR itraconazole, VRC voriconazole, AmB amphotericin B, CAS caspofungin, MCF micafungin, ANI anidulafungin, GM geometric mean, S susceptible, WT wild-type.

a. MIC modes are shown in boldface.

b. Except for fluconazole and itraconazole, results were interpreted based on the breakpoints/epidemiological cut-off values of *Candida albicans*.

c. *Pichia kudriavzevii* (= *Candida krusei*) is assumed to be intrinsically resistant to fluconazole, regardless of the MIC value.

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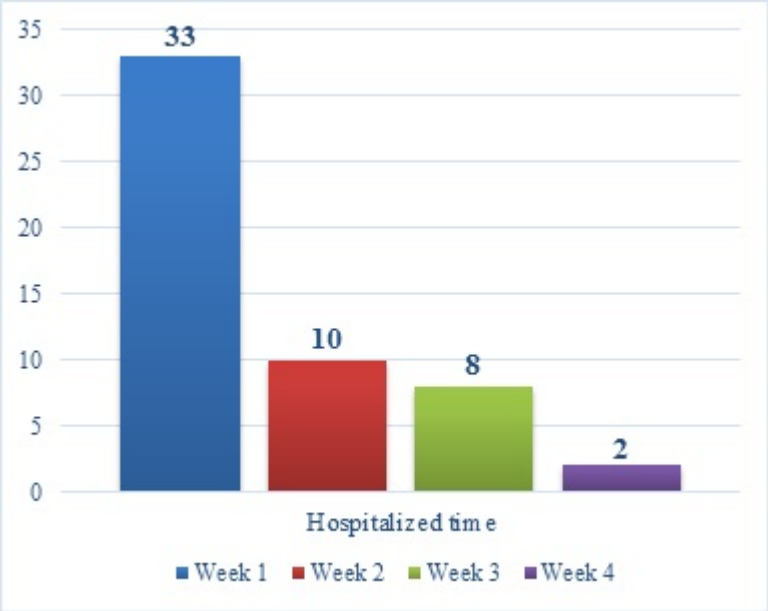

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